IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

IN RE:	Patent Application Serial No. 09/458,988	
FILED:	December 10, 1999	
		GROUP ART UNIT: 1643
TITLE:	EIA FOR MONITORING LEGIONELLA PNEUMOPHILA PRESENCE IN WATER SAMPLES	
		EXAMINER: Ja-Na Hines
APPLICANTS:	Norman James Moore Myron David Whipkey James William Welch	

BRIEF ON APPEAL

This brief is filed on the first business day following October 11, 2003. The appeal was taken April 11, 2003 from an Office Action dated October 11, 2002. A request for extension of time with requisite four month fee for a small entity and the appeal brief fee accompany this brief.

A final rejection was mailed herein on August 1, 2001, to which applicants responded on November 1, 2001, paying all requisite fees and seeking continuing examination. A non-final action was mailed January 16, 2002 and responded to on May 16, 2002 with request and

payment for a one-month time extension. On June 7, 2002 this amendment was held non-compliant and required to be resubmitted, which was done on July 8, 2002. A further non-final action mailed October 11, 2002 was appealed from, with requisite appeal and time extension fees, on April 11, 2003.

A. REAL PARTY IN INTEREST

The applicants herein have assigned all right and title in this application to Binax, Inc. of 217 Read Street, Portland, Maine 04103, a corporation of the State of Delaware.

B. RELATED APPEALS AND INTERFERENCES

There are no related interferences. There is no interference involving any patent application which Binax, Inc. owns or has the right to have assigned to it.

Application Serial No. 09/443,211, in which a notice of appeal was filed on March 26, 2003 and an appeal brief was filed on September 26, 2003 is the only other application owned by Binax, Inc. or to which it has a right of assignment that is now on appeal. The subject matter of that appeal is not related to this one.

It is of importance, however, that this application is a continuation-in-part of copending, commonly assigned U.S. patent application Serial No. 09/139,720, filed August 25, 1998, the entirety of the disclosure of which is incorporated herein by reference. Application Serial No. 09/139,720 is currently awaiting further action in the Group Art Unit from which this appeal was taken.

C. STATUS OF CLAIMS

As filed, this application had 9 claims. In the November 1, 2001 response to the final rejection mailed on August 1, 2001, these claims were all cancelled and new claims 10-35 were presented. The Office Action of January 16, 2002 made rejections to certain of these claims, as a result of which claims 10-11, 15-20, 21, 23, 25-27 and 30-35 were amended in the amendment first submitted May 16, 2002 and resubmitted July 8, 2002 in response to a "notice of non-compliance". It is claims 10-35 in their entirety, as amended on May 16, 2002 and resubmitted on July 8, 2002 that are before this Honorable Board on this appeal.

D. STATUS OF AMENDMENTS

The last Office Action mailed October 11, 2002, from which appeal was taken on April 11, 2003 entered all the amendments to claims 10-35 that were submitted on May 16, 2002 and resubmitted on July 8, 2002.[1]

Applicants have not responded, prior to this appeal, to the action mailed October 11, 2002 from which appeal was taken.

[1] The October 11, 2002 Office Action at p.2 refers to amendments made "April 8, 2002 and May 16, 2002", an obvious inadvertency. Applicants made no communication in this application on April 8, 2002 but *did resubmit* the May 16, 2002 amendment which was held noncompliant in an Office letter dated June 7, 2002 because a marked up version of the claims with handwritten rather than typed amendments had been submitted. The response was accordingly resubmitted July 8, 2002 with a marked-up typed version of the amended claims.

E. SUMMARY OF INVENTION

In parent U.S. patent application Serial No. 09/139,720[2] of which this application is a continuation-in-part, a general method is disclosed for rendering polyclonal antibodies to a carbohydrate antigen that is characteristic of a species, or serogroup of a species, of *Legionella* bacteria antigen-specific. The method involves separating the target carbohydrate antigen in essentially protein-free form from a culture of the *Legionella* bacteria species, or serogroup of a species, of which it is characteristic, coupling this carbohydrate antigen in essentially protein-free form to a chromatographic affinity column and passing polyclonal antibodies raised in an animal, such as a rabbit, against the bacterial species, or serogroup of a species, (or against the carbohydrate antigen *per se*)over the column, thereby greatly enhancing their specificity. See 09/139,720, p. 4, lines 7-15; see also Examples 1-8, pp. 8-16. This disclosure of the parent application includes the description of a 15 to 20 minute immunochromatographic ("ICT") test for detecting the crude carbohydrate antigen in fluid samples, especially samples of human bodily fluids. The test has since been approved by the FDA for detecting the O-carbohydrate antigen of *Legionella pneumophila* serogroup 1 in human urine.

In essence, this test is run on a preprepared ICT strip contained in a disposable, hinged, book-like plastic device. The strip is preprepared by (a) movably depositing labelled antibodies (which have been affinity-purified by passing them over the above-described

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[2] A copy of copending Application Serial No. 09/139,720 is attached as Appendix B hereto. This Section E is annotated to portions of that application as"09/139,720 p.__, l__". The Argument Section H is also annotated to that application where appropriate. Both this Section and Section H also contain annotations to the present application, as "this appl., p.__, l___", insofar as the specific environmental water assay disclosed herein is discussed.

chromatographic affinity column to which has been coupled the essentially protein free O-carbohydrate antigen characteristic of the *Legionella* species, or serogroup of a species sought to be detected) at a point near the sample introduction site on the ICT strip and (b) immovably binding a stripe of the same antibodies across the ICT strip near its opposite end to form a "capture" line (also called sample line). Then a sample of, e.g., urine, from a patient exhibiting *overt clinical* symptoms of an acute respiratory disease is introduced to the ICT strip and the booklike device containing it is closed so that the sample is deposited on the strip, picks up the movable deposit of labelled antibodies on the strip near the sample introduction site and the two flow together immunochromatographically toward the stripe of unlabelled immovable antibodies. If the target antigen is present in the sample, it binds to the labelled antibodies as they flow together with the sample along the strip and forms labelled antibody-antigen conjugates, which then bind again to the immovable antibodies on the capture line to form "sandwiches" of labelled antibody-antigen-immovable antibody. This causes the label material to mass along the capture line and form a distinct colored stripe. See 09/139,720, Example, VII and VIII, pp. 12-16. *Legionella pneumophila* serogroup 1 is known to cause about 80% of the Legionnaire's disease that occurs worldwide. The rapid ICT test, which can be and is successfully performed without special equipment by persons having no laboratory training has been extremely successful because it provides a quick, simple means by which physicians can (1) readily distinguish Legionnaire's disease from other bacterially caused respiratory system diseases that present very similar clinical symptoms (such as pneumococcal pneumonia and influenza) and (2) accurately prescribe appropriate medication early in the onset of the disease.

Application 09/139,720 also discloses using the ICT test to detect a *Legionella* species, or serogroup of a species, in water samples, but later testing showed that this assay was not sufficiently sensitive to detect bacteria in the concentrations found in environmental water samples. In this connection, "environmental water" encompasses high sediment standing water in heating and cooling systems and in stagnant outdoor ponds etc., and also low sediment water that has been flowing through pipes, such as drinking and bathing water (this appln., p.1, lines 1-5 and p. 5, last four lines to p. 6, lines 1-6). *Legionella* bacteria typically breed and grow in still water and in pipes which have been in use for a long time. For public health and safety, the detection and elimination of these bacteria in standing water and in water pipes is of importance, because this detection has the potential for eliminating the bacteria by treatment at their breeding sites and thereby eliminating, or greatly reducing, *Legionella*-caused human disease. In addition, OSHA has established a "safe" concentration level of such bacteria and it is important for water in buildings to be monitored to be certain that water delivered to people who live or work in them remains below the safe concentration.(this appln., p. 1, line10-p. 2 line 9)

The present application discloses and claims a highly sensitive enzyme immunoassay employing antibodies to O-carbohydrate antigens of *Legionella* that have been treated by the affinity purification methodology of the parent application, and using them to detect the target characteristic carbohydrate antigen of *Legionella* that is present in the living and nonliving *Legionella* bacteria that inhabit and breed in environmental water. (this appln., p. 4, lines 1-22)

The method involves coating a solid substrate (e.g. a test tube) with the antibodies treated as described in the parent application, preparing a conjugate of an enzyme and another increment of the treated antibodies, contacting 0.2 to 2.0 μg per ml of this conjugate with sample of environmental water suspected of *Legionella* contamination, a buffer and the coated solid substrate, in a vessel (which may be the coated test tube), incubating for at least 20 minutes, decanting the vessel, adding a colorimetic, chemiluminescent or bioluminescent material to the vessel, and allowing it to stand for up to 5 minutes, followed by reading the color, chemiluminescence or bioluminescence intensity in a suitable instrument and correlating the concentration of bacteria present in the sample to the intensity reading using a standard prepared in a manner well known to those skilled in immunochemistry. See the present application, "Brief description of the Invention, pp.4-5; see also Example 2, p.11, l.1 to p.13, l.12.

Because the water samples must be collected in substantial volume to perform the test accurately, an immunoconcentration step is necessary prior to conducting the test. Sample collection and immunoconcentration procedures are described in the application at p.5 under the heading "Detailed Description of the Invention", commencing in the 6th line under the heading and continuing on page 6, ending at line 3, p.7. The methods include filtration, centrifugation and concentrating with magnetic microparticles which have been coated with the purified antibodies.

F. THE ISSUES

The major issue here is whether applicants may properly incorporate by reference the entirety of the disclosure of parent Application Serial No.09/139,720 into this application, including material therein that is essential to the present invention, particularly (1) the mode of obtaining the characteristic O-carbohydrate antigen of a *Legionella* bacteria species, or serogroup of a species, from a culture of the same bacteria in essentially protein-free form (i.e. containing not more than about 10% of protein on a wt/wt. basis) (2) the description of the coupling of the thus purified antigen to a chromatographic affinity column and the use of that column in purifying polyclonal antibodies, raised in an animal against the bacterium or the antigen *per se*, to render these antibodies highly antigen-specific. The new matter rejection under 35 U.S.C §112, first paragraph, arises because it is the Examiner's position that essential material is missing from the application and it cannot be incorporated by reference. The rejections stated in paragraphs 5, 10, 12, 13, (in part) 14 and15 of the action appealed from all result, in one way or another from the incorporation by reference issue.

A second issue here of double patenting, is set out in numbered paragraphs 11, 16 and 17 of the action appealed from. It involves alleged overlap between the claims of this application and its parent application Serial No. 09/139,720 or between this application and a continuation-in-part, *inter alia*, of both this application and parent application Serial No. 09/139,720. This copending, commonly assigned continuation in part application is Serial No. 518,165, filed March 1, 2000.

A third issue involves specific alleged indefiniteness under 35 U.S.C. §112, second paragraph. It applies to claims 10, 25, 21, 32 and 25 and is set out in numbered paragraphs 6-9 inclusive. In the main it involves specific rejections based on specific criticism.

A fourth issue is an enablement issue that is *new* in this case with this action dated October 11, 2002 and is set forth in numbered paragraph 13 of the action. It is a rejection the same Examiner had previously equally belatedly raised in parent application 09/139,720 and in continuation-in-part application 09/518,165. It involves assumptions made by the Examiner and arbitrarily asserted without any support for them, to **wit**:

1) an assumption that unless a process is performed in one continuous, ongoing operation it cannot be patented.

2) an assumption that the Examiner may extrapolate from the fragmentary portion of "Critical Synergy: The Biotechnology Industry and Intellectual Property Protection (more elaborately cited at p.10 of the action) which relates solely to claiming of DNA segments and claiming problems relative to purification processes for obtaining proteins purified to 2 or 3 decimal places, neither of which is presented here.

The extrapolation involves an arbitrary requirement for a rigor of infinite detail in both the disclosure and claims relative to operations that are routine in the art to immunologists, made on the assumed, unsupported premise that "highly empirical" operations are involved here.

A feature of this "enablement" rejection as raised herein for the first time in the second action following final rejection is its capricious and pnecemeal character and the lack of support for it.

It is also noted that paragraph 2 of the action appealed from contains a requirement for new drawings which has not been complied with in view of the decision to appeal. It is respectfully requested that this requirement be held in abeyance pending return of this application to the Group Art Unit, whereupon Applicants will endeavor to make timely compliance. Applicants' counsel sincerely apologizes for failing to deal with this matter earlier.

G. GROUPING OF CLAIMS

Of the claims 10-35, claim 10 is an independent claim upon which each of claims 11-24 depends, directly or indirectly and claim 25 is an independent claim upon which each of claims 26-35 depends, directly or indirectly.

If claims 10 and 25 are each held patentable, the patentability of each of their respective dependent claims necessarily flows from that holding.

H. ARGUMENT

The first issue to be resolved is that of whether Applicants are entitled under the existing state of the law, to incorporate by reference the entirety of the disclosure of parent application Ser. No. 09/139,720 which is copending and commonly assigned, into this application 09/458,998. If *so*, those of the rejections that are based upon treating subject

matter fully disclosed in the incorporated by reference parent application as "new matter" in the claims of this application, or as missing from this application and rendering the claims unenabled necessary must also be overruled.

1. Incorporation By Reference, Alleged New Matter, Allegedly "Missing" Essential Material and Enablement

What superficially sound like several grounds of rejection here are just different ways of saying the same thing. The Examiner, having adamantly ruled that "essential material"cannot be incorporated by reference in this application but must be inserted by an amendment supported by affidavit or declaration in accord with *In re Hawkins* 179 USPQ 157 (CCPA 1973); *In re Hawkins* 179 USPQ 163 (CCPA 1973) and *In re Hawkins* 179 USPQ 167 (CCPA 1973), *all* of which relate only to attempted incorporation by reference from a foreign application, and *none* of which even remotely discusses incorporation by reference of all the disclosures of a copending, commonly assigned U.S. parent application into a continuation-in part thereof, which is what has been done here.

The Examiner cites *nothing* to support the position taken except the *Hawkins* cases, which are clearly not in point. Applicants' counsel has repeatedly brought to the Examiner's attention the "Incorporation By Reference" discussion in M.P.E.P. 608.01 (p) commencing at the page numbered 600-79 dated August 2001. On that page, after pointing out the wide latitude of discretion that the Commissioner (now Director) has in establishing and maintaining incorporation by reference policy, the Manual points out under the ensuing A subheading in the first paragraph that:

> "An application as filed must be complete in itself in order to comply with 35 U.S.C. 112. Material nevertheless may be incorporated by reference. *Ex parte Schwarze*, 151 USPQ 426 (Bd. App. 1966). An application for a patent when filed may incorporate "essential material" by reference to (1) a U.S. patent, (2) a U.S. patent application publication, or (3) a pending U.S. application, subject to the conditions set forth below."

The M.P.E.P. in the next paragraph goes on to state that "'Essential material' is defined as that which is necessary to (1) describe the claimed invention, (2) provide an enabling disclosure of the claimed invention or (3) describe the best mode (35 U.S.C. §112)". In essence, the M.P.E.P. thus endorses the Applicants' position that the rejections of the claims herein as containing "new matter" and as "nonenabled by the disclosure" or as "missing essential material" *all* spring from the refusal of the Examiner to permit the incorporation herein by reference of Application Ser. No. 09/139,720 in its entirety--an incorporation made on the first page of this application and retained therein pending a ruling on appeal, even though repudiated by the Examiner.

The situation is particularly difficult, given that the Examiner has no reference or reference combination that in any way reflects adversely on the claims of either parent application Ser No. 09/139,720 oor the claims of this application, yet persists in holding up the allowance of any claim in either on capricious, wholly synthetic, grounds that have no basis within the bounds of the relatively mature science of immunology.

It should further be recognized here that the disclosure (but *not* the claims) of parent application Serial No. 09/139,720 has been incorporated by reference herein in its entirety. The reason is that the parent application generically discloses, *inter alia*, that polyclonal antibodies raised to a *Legionella* bacteria species, or serogroup of a species, (or to the target

carbohydrate antigen of a *Legionella* species or serogroup of a species) and affinity purified as disclosed therein are especially efficient and sensitive detection agents for the crude O-carbohydrate antigen of the same *Legionella* species, or serogroup, whether it is present in live bacteria or dead ones, in whole bacteria or fragmentary ones or in free form in human urine, blood or other bodily fluid and that they can consequently be made the detection agent in a great variety of known assays (See, e.g., Ser. No. 09/137,280, p.3, lines 12-15). The disclosure in the parent application of the preferred rapid ICT assay for detection of the O-carbohydrate antigen characteristic of a given *Legionella* species, or serogroup of a species, is itself a species of the various known, common types of immunological assays in which these antibodies are capable of being substituted for raw polyclonal antibodies of the prior art, with improvement of assay results. The particular enzymeimmunoassay ("EIA") described and claimed herein, realistically, is another species of common immunoassay procedure to which improved results have been imparted by modifying it to employ as detection agents antibodies that have been affinity purified according to the disclosure given in the parent application and incorporated herein by reference. The two applications may, together, thus be viewed as a continuum.

The Examiner's first "new matter" rejection set out in numbered paragraph 5 of the rejection appealed from particularly results from the insistence that incorporation by reference of the parent application disclosure will not be permitted. Initially, the present application was presented with more general, brief claims which defined the antibodies, as "antigen-specific antibodies obtained by purifying raw polyvalent anti-*Legionella pneumophila* serogroup 1 antibodies on a chromatographic column to which is coupled a conjugate of an essentially

protein-free polysaccharide antigen of *L. pneumophila* and a spacer molecule".

Through two actions, the second of which mailed August 1, 2001 was a final rejection, the Examiner insisted that "the claims of the instant application *need to claim* the same antibodies and procedures as found in 09/139,720." (August 1, 2001 final rejection p.3; Emphasis added) It is also noted that the Examiner *in that* Action specifically stated that "It is understood that 09/139,720 is incorporated by reference." (*id*).

Based upon this position stated by the Examiner, Applicants presented essentially the same claims 10-35 that are now on appeal[3], which are broadly patterned on claims now present in parent Application Serial No. 09/139,720,insofar as the recitation of the steps by which the antibodies used as detection agent are pretreated, is concerned. At the same time, claims 1-9 were cancelled.

Shortly after receiving the claims in the form required by the August 1, 2001 final action, the Examiner switched positions and commenced (a) holding incorporation by reference to be improper and (b) rejecting Claims 10-35 as containing "new matter" and as containing subject matter not described in such a way as to be enabling (See January 16, 2002 action number paragraphs 4-6 and 15).

The same rejections directly contradictory of the August 1, 2001 final action are being retained here.

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[3] Slight modifications to these claims as first presented have been made in response to the January 16, 2002 office action, but the basic framework of claims 10-35 is unchanged.

Applicants are being whipsawed between two utterly conflicting viewpoints regarding how to claim the present invention--one adopted initially and in the final rejection, the second diametrically opposite one being espoused in the two actions of January 16, 2002 and the one appealed from dated October 11, 2002.

Applicants' counsel has repeatedly striven to co-operate with the Examiner and move this clearly patentable invention toward allowance, only to be met consistently with new rejections that are capriciously inconsistent with the previous ones.

Applicants believe that the incorporation by reference of the entire disclosure of Application Serial No. 09/139,720 herein is in strict compliance with the M.P.E.P. and the practice defined in apposite case law and urge that this Honorable Board so rule. As a practical matter, Applicant believes that simultaneous allowance and issuance of *both* Application 09/139,720 and Application Serial No. 458,998 would be a very effective way of putting an end to the whipsawing effect seen in the diverse, often unfounded §112 rejections that characterize the seemingly endless prosecution procedure that has been compelled in both applications..

Returning to the action appealed from, Claims 10-35 are mischaracterized in that action as "drawn to a method for determining the concentration of at least one species or serogroup of a species of *Legionella* bacteria in a fluid..." (Action p.3, ¶5.) The two independent claims here, 10 and 25 are restricted respectively to making that determination "in water" (claim 10) and "in environmental water" (claim 25). The Action asserts that there is "no teaching of a wet cell pellet" (*id.*) in this application, "no support... for separating the mixture or removing the upper layers" (*id.*) "no support for use of broad spectrum protease" (*id.*) or "separating

out an essentially protein free carbohydrate," no support for obtaining an essentially protein-free carbohydrate antigen by a series of steps now claimed" (*id.*) The *support* for each is in Application 09/139,720, however, as has been painstabkingly and laboriously pointed out in the long and arduous prosecution of that application. Moreover, the final rejection of August 1, 2001 herein clearly directed that *all* of the details of the 09/139,720 claims that are essential to the purification of antibodies to *Legionella* bacteria be spelled out herein *because* of the incorporation by reference. By then doing an aboutface in the January 16, 2002 action, continued in the October 11, 2002 action appealed from, incorporation by reference is now held to be improper and the claim content Applicants were *directed* to include herein is declared "new matter".

Furthermore, the action appealed from incorrectly states that "the specification [apparently of this application 09/458,998] states that the use of raw polyclonal antibodies is an essential element in the performance/sensitivity of the assay" (p.4, 3rd paragraph). No such statement, however can be found in the specification of the application and no reference is provided. The last sentence of the same paragraph asserting "the claims encompass new matter by not limiting [them] to the purified raw polyclonal antibody which are an essential element" is not only hard to understand but *ignores* that all of the steps by which the parent application, incorporated by reference here, describes purifying "raw polyclonal antibodies" are present in claims independent 10 and 25 as a result of the Examiner's direction in the August 1, 2001 final rejection, that this be accomplished.

Applicants and their counsel stress that their object here is to cooperate in describing the invention in the manner required by the statute and the rules and that they have endeavored to meet the Examiner's rejections and objections with courtesy and attention. This appeal arises, however, because applicants have come to conclude, reluctantly, that the prosecution process is not working in this application and that somehow, some further direction is needed to make it work. The same rejection present in ¶4, pp.2-4 is in part reiterated throughout numbered ¶12 (pp.8-12) with additional elaborations, and briefly in ¶13 as "a new matter rejection". *If*, as applicants believe, the incorporation by reference of all the disclosure of parent application 09/139,720 is proper here, these "enablement" and "new matter" rejections are improper. The Board is respectfully requested to clarify this situation.

Applicants note that one simple and relatively clear resolution of the situation *could* be made if independent claims 10 and 35 could be simplified to adopt a definition of the purified antibodies that encompasses the purification procedure described fully in the parent application. For example, if Claim 10 were rewritten to eliminate all of steps (a)-(d) and present step (e) (i) were rewritten in its first two lines to recite "coating a solid substrate with purified antigen-specific antibodies obtained according to the process described in parent application Ser. No. 09/139,720", a relatively simple independent claim could be fashioned. Since this precise mode of describing the antibodies in the claim is believed inappropriate under present practice, Applicants propose that the term "antigen-specific antibodies" could be defined in the specification of this application by inserting a sentence saying "By purified antigen-specific antibodies' as used herein and in the ensuing claims, is meant antibodies to the characteristic O-carbohydrate antigens of the *Legionella* bacteria species, or serogroup of a

species, to be detected that have been affinity purified in the manner described in detail in parent Application Ser. No. 09/139,720". Such a sentence would not be new matter, both because of the incorporation of the parent case by reference and because at least the Examiner, the Applicants, and counsel are believed to have understood from the outset that this defines what has been and is being talked about throughout this application. Inserting such a definitive sentence would permit the two independent claims not only to eliminate steps a-d inclusive now recited therein, but also to use the term "purified antigen-specific antibodies" in what is now step (e) (i) of both independent claims in lieu of "purified antigen-specific antibodies from step (d) hereof".

It is also believed that the clarity of the claims would benefit from the proposed simplification, were the Board to deem such a procedure appropriate to this situation.

2. "Double Patenting"

The "double patenting" rejections of numbered paragraphs 11, 16 and 17 used little discussion here, because they are presently not ripe for decision-making. Applicants' assignee stands ready, when and if it becomes certain which of the three, commonly assigned, copending applications, i.e. Ser. No. 09/139,720, Ser. No. 09/518,165 and this one, will issue first, to make terminal disclaimers in each of the remaining two identified applications, ensuring that all three will expire simultaneously.

The rejection set forth in ¶11 of the action appealed from suggests that this rejection, based on the present claims of Application Ser. No. 09/139,720 is, in any event, not well taken and can be resolved. The differences between the ICT assay claimed in Ser. No. 09/139,720

and the enzymeimmunoassay claimed herein are sufficiently clear and distinct that a line of demarcation is believed to be readily attainable if needed.

The double patenting rejection set forth in paragraph 16 of the action is one that also allows for the possibility of developing a line of demarcation, which can be handled when it is clearer which of the applications is likely to issue first and what demarcation is needed.

The double patenting rejection set forth in paragraph 17 of the action appealed from is in the same posture as that of ¶11 and should not be a basis for action by this Board at this time when issuance of all three applications remains unsettled. If this application issues first, claims in the other applications can clearly be reformulated to avoid overlaps if necessary. Moreover, in the case of obviousness-type double patenting, the filing by the common assignee of appropriate terminal disclaimers at the appropriate time(s) should solve the problem completely.

3. Indefiniteness Rejectious of Paragraphs 6-9 and 15

These rejections are largely of a trivial nature which, however, deserve resolution.

Paragraph 6 says that Claims 10 and 25 are unclear because O-carbohydrate antigen is not mentioned until the respective portions (e) (iv) of each of these independent claims and it is "unclear how to correlate O-carbohydrate antigen in the sample to CFU of *Legionella* bacteria in the water originally sampled.

> *In fact*, claim 10, in its line 3 recites that the method "detects O-carbohydrate antigen". This is the antecedent for the recitation in step (e) (iv) of the same claim for "the amount of O-carbohydrate antigen....detected in the sample...." Claim 25 is in the same posture as Claim 10.

Further, it is believed that the mode of correlation is quite clear in the claim and in the application. Intensity concentration standards have been developed for this method by availing of to well-known, frequently used of, routine procedures of immunology wherein samples of known concentration are run in tests where color, chemiluminescence or bioluminescence is measured and reference standards are. From these reference standards, the intensity of measured color, chemiluminescence, or bioluminescence is directly correlated by a human being to the concentration and amount of the O-carbohydrate antigen in the sample that produces that degree of color, chemiluminescence or bioluminescence measured with the sample. Standards that correlate the amount of the O-carbohydrate antigen in the sample to CFU/ml. of bacteria in the water are also predetermined in the same way and are then consulted to "correlate that amount in turn to the concentration of the suspected Legionella bacteria...in the water being tested." See also p. 8 of the application, lines 11-18.

In this numbered paragraph 5, at p. 5 of the action, the Examiner resorts to an assumption *unsupported* by this application or parent application 09/139,720--that the antibodies used in this application will not detect the crude O-carbohydrate antigen in a sample, but only will detect such an antigen that is protein-free. This *incorrect* assumption, which has surfaced repeatedly during the prosecution of this application and its parent Serial No. 09/139,720 is refuted by the hundreds of thousands of ICT tests which applicants' assignee has distributed around the world, all of which have detected a crude carbohydrate antigen in various samples, the many enzymeimmunoassay test kits that have also been distributed for testing of environmental water, and have successfully detected crude carbohydrate antigens in

samples and the fact that it would be out -and -out ridiculous to develop a test that could *not* detect a target antigen in a sample unless and until the antigen were first removed from the sample and treated to render it protein-free. The ICT tests for *Legionella pneumophila* serogroup 1 that applicants' assignee manufactures have been FDA-approved to detect the O-carbohydrate antigen of that bacterium in human urine. These tests are designed to be run by anyone who can read and comprehend the directions, at any site, without any special equipment. The request for "clarification...as to what the antibodies are specifically binding" (Action p.5, par.6) defies common sense and the implied rejection should be overruled.

The rejection of Claim 21 in paragraph 7 of the action appealed from, for failure to define the buffer used is capricious. It is well-known in immunology that various common buffers may be interchangeably used in a variety of operations without in any way affecting the outcome of those operations. Dependent claim 22, which is dependent on claim 21, recites a particularly preferred buffer to be used in the process of claim 21. Claim 21, however, should *not* be limited to a particular buffer when numerous others will serve the purpose. The point of the rejection here is not to obtain disclosure of suitable buffer reagents given that one is specifically claimed in dependent claim 22, but to deny Applicants reasonable claim scope consistent with the ordinary level of skill among immunologists, to Claim 21. The rejection is ill-taken and should be overruled.

The rejection of Claim 32 in numbered paragraph 8 does not make sense. Claim 25 in step (f) (iii) recites the use of a test reaction vessel, which vessel is also present in step (f) (iv) where it is decanted, washed and treated with a colorimetic, chemiluminescent or bioluminescent material which develops intensity proportional to the antibody-antigen reaction

products formed on the vessel walls or on a solid insert enclosed by the vessel Claim 32 *properly* refers to the test reaction vessel present in steps (f) (iii) and (f) (iv) and the rejection is without merit.

The rejection of Claim 25 in paragraph 9 is likewise capricious. It rests upon the absence from step (e) of Claim 25 of the word "contacting". The claim recites that when antibody-coated magnetizable particles and *Legionella* bacteria-containing water samples are mixed, the antibodies coated on the particles tend to draw to themselves O-carbohydrate antigens in the bacteria and react with them to form conjugates. *Contact* is *inherent* in the formation of these antibody-antigen conjugates and any argument to the contrary is plainly frivolous. The claim is proper and the absence of the word "contact" or "contacting" is of no moment and no legal significance. This rejection, too, is without merit.

4. The 35 U.S.C. §112 New Grounds for Rejection Set Forth in Paragraph 12 of the Action

This rejection is patterned upon one made in both parent Application 09/139,720 and in c-i-p application 09/518,165.

The first premise of this rejection is that there is something *per se* unpatentable about a method claim, the steps of which either cannot be performed in one unbroken operation, or that are exemplified in separate examples of a patent application. No case, treatise or authority to that effect is cited, and it is certainly believed by Applicants that none exists.

The idea of a prohibition against covering, in one "hybrid" claim, all the steps needed to obtain the antigen-specific antibodies of this invention and employ them in an ICT assay designed to discover from a patient sample of fluid (e.g. urine, blood) whether the patient is

infected with *Legionella* bacteria that contain the antigen to which the antibodies have been made specific seems designed only to prevent inventors from covering *all* of what they have invented in cases where complicated methodology is needed. Applicants submit that the rejection premised on this notion has no basis in statute, rule or case law and, further, is of no practical utility in carrying out the objectives of the patent laws.

The paragraph of numbered "paragraph" 12 on pages 9-10 of the action is repetitive of the content of numbered paragraph 5 which has been addressed in Section 1 of this Argument Section H and is not rediscussed here.

Commencing in the first full paragraph of "paragraph" 12 on page 10, a rejection new to this case is set out, which rejection was made previously in copending parent application Serial No. 09/139,720 and copending c-i-p application Serial No. 518,165. This rejection is based upon the "Critical Synergy" fragmentary publication mentioned above. It should be overruled because it has no place in this application, is without merit and is an effort to impose standards of disclosure needed in new technologies that are as yet unsupported by a substantial body of literature upon mature technologies in which the level of skill in the art is well developed and backed by an extensive body of literature.

The document cited at page 12 of the action comprises pages 100-107 of what is obviously a much larger document of *at least* 107 pages. The fragment of it relied upon in the action does not mention immunochemistry, antigens, antibodies, assays, affinity purification techniques or anything else directly connectable to this application. Its author or authors are unknown. Its recommendations have not been adopted as a standard for measuring the disclosure of immunochemical patent applications, nor are they likely to be, since the fragment does not

discuss any aspect of immunochemistry.

Applicant's counsel has endeavored, over a period in excess of one year, to obtain a copy of this entire "publication", because the fragment cited in the action and made available to such counsel by the Examiner is clearly taken out of context and geared to specific technology areas that are non-inclusive of the field of immunochemistry with which this application is concerned. Standards of disclosure and of level of skill in the art in immunochemistry are fairly well established, having been developing since at least the mid- 1960's. This immunological technical area is wholly unlike either gene identification or protein purification to extremely high degrees of purity, which seem to be the major topics of the fragment. Moreover, the fragment is presented from a strongly partisan viewpoint but the identity or identities of the speaker(s) or writer(s), whichever they maybe, is concealed.

In the immunochemistry field, including immunoassay and antigen -antibody work, persons of ordinary skill in the art have a *large* body of knowledge concerning equivalence of various reagents, buffers, columns, lyophilization techniques and the like. They also have a large body of knowledge about what is nonequivalent. The interchangeability, e.g., of centrifugation with allowing a mixture to settle naturally and then decanting or aspirating or syphoning off an upper layer with other common laboratory techniques for separating two layers of liquid, is well known in chemistry generally and also in immunochemistry. Workers in these arts recognize all of these techniques as mere routine manipulations that do *not* affect the chemical compositions of the layers and that certainly do *not* require infinite descriptive detail to be thoroughly understood.

The unceremonious and unprecedented comparison in this action of these and other commonly understood aspects of immunochemistry, including antigen-antibody work, to what may (or may not, given the apparent bias of the unknown writer(s) of the Critical Synergy fragment) be still under exploration in intensive protein purification and/or gene identification is apparently intended to impose upon those in the immunochemistry field, an unprecedentedly high standard of disclosure of infinite detail that then imposes an equally restrictive amount of detail upon claim terms, all accomplished without any change in the level of disclosure or claiming accepted by the Patent and Trademark Office *per se* and its examining corps in general. Doing this without advance warning that standards are to be changed and made more restrictive than ever before, and without opportunity for public discussion followed by rule-making, is inconsistent with due process of law and totally unfair to applicants accustomed to greater understanding of the level of ordinary skill in their own particular field of endeavor. Its primary effect, if indulged be to force claims into such detailed specificity that a patent owner's competitors would find it easy to appropriate its patented inventions without infringing its patent claims.

The Action's reliance on the Synergy fragment supplied is particularly questionable as a matter of law, moreover, when the following facts are considered:

1. Initial inquiry for this entire document at the Biotechnology Industry Organization which, according to the office action, "published" the whole of the cited document, elicited the information that the document is unavailable because "it's too old". Further searching inquiry over several phone calls made by 2 or 3 different people established that the alleged "publisher" has no copy, cannot

identify anyone else who does and doesn't consider that it has any responsibility for assisting anyone to find it.

2. Efforts were then made to find the whole document by inquiries to each of the following, all of whom stated they had no copy and some of whom said "it's not published" or "there's no such publication", after looking for it and finding no record of any sort regarding it:

1. Patent Depository Library, San Diego, CA
2. U.S. Department of Commerce Library
3. Patent and Trademark Office libraries and offices as follows:
   a) Biotechnology Library
   b) Scientific and Technical Information Center
   c) Office of the Solicitor
   d) Patent and Trademark Office Library
4. Library of Congress
5. U.S. Senate Library
6. Canada Institute of Science & Technology
7. National Library of Medicine
8. LEXIS
9. Westlaw
10. British Library
11. Georgetown University Law Library
12. Federal Depository Library
13. Linda Hall Library of Science, Engineering and Technology

We emphasize that this investigation did not merely involve failure to find it on a library shelf; or even find it in a library catalogue, but involved direct communication with knowledgeable personnel who made what they represented to be serious efforts to locate such a "publication".

From all of this, it can only be concluded that, whatever the motive or inspiration for reliance on this out -of -context fragment from a purported whole document of *at least* 107 pages may be, it is misplaced and the fragment should be laid to rest in the same way that the apparent original sponsor, the Biotechnology Industry Organization, has already fully buried it.

The rejection of the claims herein on the apparent ground that one skilled in the art of immunochemistry techniques would be incapable of practicing the claimed process of the independent claims 10 and 25 because the first step involves culturing a bacterial species or serotype of a species and it continues through obtaining an essentially protein-free carbohydrate antigen, coupling the antigen to a chromatographic affinity column molecule , passing antibodies over the column and then utilizing the resultant antigen-specific antibodies in an assay to detect the crude carbohydrate antigen is based upon a number of assumptions that are not at all clear. The rejection, as based on the "Synergy" fragment is misplaced and should be overruled.

The action, in ¶12 on page 11 contains two additional, already many times discredited rejections based on wrong assumptions made by the Examiner. Application 09/139,720 does *not* disclose, anywhere, that the solution (provided in every kit of Binax's NOW® ICT test for identifying *Legionella pneumophia* serogroup 1 in samples of human urine) cand called "Reagent A" on p.15 thereof, is required" to produce the crude carbohydrate antigen" (Action appealed from, p.10). The *assumption* that this is the fact is the Examiner's, and it is mistaken. *That* "Reagent A" contains Tween 20, sodium azide and sodium dodecyl sulfate in

sodium citrate buffer. *Two drops* of it are added to human body fluid samples at the commencement of the ICT test to promote flow of the sample plus the movably deposited conjugates of colloidal gold and antigen specific antibodies placed on the strip in the sample flow path toward the "capture line" of the ICT test. It should be recognized that, as the Examiner has many times been advised, *Legionella* bacteria that have passed through the human kidneys into urine samples have no need to have their cell walls opened up because this opening up occurred during the physiological-chemical processes of the human body, including the kidneys, before the bacteria ever passed into human urine. See, e.g. p.3 of this application, the sentence at lines 10-13.

This application, itself deals with an assay conducted in, e.g., a test tube where flow is not a consideration in the process and there is *no* need for a flow promoter. In conducting the assay of this invention, it should be recognized that the cell walls of the bacteria, many of which are live, *do* need to be broken open in many cases. The specification points, out that this may be done mechanically by stroking with a swab pad (p. 12, lines 15-16), or with the aid of a different "reagent A" consisting of SB 3-8, a zwitterionic detergent, in Tris base (p. 9,lines 13-18). *Neither* the use of this "Reagent A" to open cell walls of live and dead whole bacteria *in a sample*, nor the use of the flow promotor "Reagent A" to encourage *sample flow* in the ICT test of Serial No. 09/139,720, has anything to do with the steps of separating an essentially protein free carbohydrate antigen from a culture of *Legionella* bacteria, however. The Examiner's suggestion (at p.11 of the action appealed from) that it does is unsupported by any reference, and inconsistent with both this application, and its parent Application Serial No. 09/139,720.

The penultimate paragraph at page 11-12 of "paragraph" 12 of the Action presents no reason or justification for limiting the antibodies recited in the claims to "purified raw polyclonal antibodies"--an oxymoron at best because" purified" antibodies, whether or not polyclonal, are *never* "raw" and "raw" antibodies are likewise never "purified". There is no question that the antibodies referred to in the claims started as raw polyclonal antibodies. When treated as described in the parent application, by affinity purification on a chromatographic affinity column having an essentially protein free O-carbohydrate antigen attached thereto, the resulting antibodies are *no longer* "raw" and are antigen-specific as well as purified. The Examiner's insistence that they be denominated "purified raw polyclonal" antibodies in the claims is misplaced. The failure to so *mis*characterize them in no sense detracts from the fact that the present claims are fully enabled. The rejection based on this false premise should be overruled.

The Examiner's final paragraph of "paragraph" 12 on p.13 of the action is way off-base in suggesting that guidance as to bacterial substrates should be provided. This overlooks that bacteria are today obtained from depositories, like American Type Culture Collection or Pasteur Institute and from entities such as Centers for Disease Control, who not only provide a complete identification of the bacteria sent but also send directions for culturing them that include specifying the culture medium ("substrate" in the Action, p.12) to be used.

I. CONCLUSION

Summing up, Applicants request that the propriety of incorporating by reference the parent application disclosure be upheld and the Examiner's "new-matter" and "nonenablement" rejections that are based upon disallowance of incorporation by reference be overruled and that the Board also consider the question raised herein, of whether a definition may be incorporated in this case that equates "purified antigen-specific antibodies" to those that have been affinity purified as described in the parent application Serial No. 09/139,720, which the quoted tern can then be utilized in the two independent claims to reduce their length and render them clearer and more readable.

Applicants further suggest that the double patenting issues, which are not yet well enough defined to be ripe for adjudication be so pronounced and remanded to the Group Art Unit.

Finally, applicants request that the rejections discussed in subsections 3 and 4 of Section H of this brief be overruled in their entirety.

Respectfully submitted,

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Attachments: Appendix A: Appealed Claims 10-35
Appendix B: U.S. Application Serial No. 09/139,720 Without Claims
Note: The copies of Appendix B provided contain a few handwritten corrections that reflect minor amendments made to the disclosure.

APPENDIX A

APPEALED CLAIMS

10. A method sufficiently sensitive to determine a concentration at least as low as from 5 to 50 colony-forming units ("CFU") per milliliter ("ml.") of water of at least one species, or serogroup of a species, of *Legionella* bacteria in water suspected of being infected therewith, which method detects an O-carbohydrate antigen characteristic of said at least one species or serogroup of a species of *Legionella* bacteria and comprises the following steps:

(a) culturing an identified *Legionella* bacteria species, or serogroup of a species, to a desired size and harvesting therefrom cells of that species, or serogroup of a species, as a wet cell pellet;

(b) obtaining from the wet cell pellet an essentially protein-free embodiment of the characteristic O-carbohydrate antigen of that species, or serogroup of a species, of *Legionella* bacteria by a series of steps which comprises

(i) suspending the wet cell pellet in an alkaline solution and mixing to form a mixture;

(ii) adjusting the pH of the mixture to an acid pH with a strong acid;

(iii) separating the mixture from step (ii) into two layers, an upper layer and a lower layer;

(iv) removing the upper layer and adjusting its pH to approximate neutrality;

(v) adding to the approximately neutral pH upper layer from step (iv) a broad spectrum protease enzyme and digesting to destroy residual proteins;

(vi) adjusting the pH of the digested product from step (v) to an alkaline pH with a dilute aqueous alkaline solution of a weak base and;

(vii) separating out the essentially protein free O - carbohydrate antigen embodiment;

(c) coupling to a chromatographic affinity column through a spacer molecule the essentially protein-free O-carbohydrate antigen embodiment obtained in step (b);

(d) passing polyclonal antibodies to the *same Legionella* species, or serogroup of a species, as that cultured in step (a) over the chromatographic affinity column of step (c) to produce purified antigen-specific antibodies; and

(e) performing an enzyme immunoassay upon a water sample suspected of being infected with *Legionella* bacteria of the same species, or serogroup of a species, as that cultured in step (a), which assay comprises the following steps:

(i) coating a solid substrate with purified antigen-specific antibodies from step (d) hereof in an amount sufficient to provide a coating containing at least 0.05 μg per test of said antibodies and allowing the coated substrate to dry;

(ii) preparing a conjugate of an enzyme and purified antigen-specific anti-bodies from step (d) hereof;

(iii) bringing the sample to be tested and from 0.2 to 2.0 μg conjugate per test into contact with a buffer solution and the coated solid substrate of step e (i) in a test reaction vessel and incubating for a period of at least 20 minutes; and

(iv) decanting liquid from said vessel, washing, adding a colorimetric, chemiluminescent or bioluminescent material thereto, allowing color, chemiluminescence or bioluminescence to develop for up to about 5 minutes, measuring its intensity in any known manner and determining therefrom the concentration of the suspected *Legionella* bacteria species or serogroup of a species, in the original water sample according to predetermined intensity/concentration standards which first correlate the measured intensity to the amount of O-carbohydrate antigen characteristic of at least said suspected species, or serogroup of a species, of *Legionella* detected in the sample and then correlate that amount in turn to the concentration of the suspected *Legionella* bacteria species, or serogroup of a species, in CFU/ml that is present in the water being tested.

11 The method of claim 10 in which the *Legionella* bacteria species, or serogroup of a species, in step (a) is a serogroup of *Legionella pneumophila* bacteria and the polyclonal antibodies of step (d) are polyclonal antibodies to bacteria of the same serogroup of *Legionella pneumophila*.

15 A method according to claim 10 wherein the enzyme immunoassay is run as a sandwich assay.

16 A method according to claim 10 wherein the enzyme immunoassay is run as a competitive assay.

17 A method according to claim 10 wherein the coated solid substrate in step (e) (i) is the inner wall of a test tube, which test tube also serves as the test reaction vessel in [step] steps (e) (iii) and e (iv).

18 A method according to claim 10 wherein the coated solid substrate of step (e) (i) is selected from among coated solid inserts and coated beads.

19 A method according to claim 10 wherein the enzyme of the conjugate prepared in step (e) (ii) is horseradish peroxidase and a colorimetric agent, tetramethylbenzidine, is added in step (e) (iv).

20 A method according to claim 10 wherein the water to be tested is water obtained from [the] a heating and/or cooling system of a building, or water obtained from a building sanitation or drinking water supply, individual water samples are obtained in quantities of from 100 to 1000 ml. per test and each individual water sample is subjected to a pre-assay concentration step.

21 A method according to claim 20 wherein the concentration step comprises filtering each individual water sample through a filter having a pore size not greater than 0.45μm, and it is followed by collecting filter residue by thoroughly stroking the filter with the swab pad of a swab comprising a handle and an affixed pad of fibrous material or foamed open pore material , and delivering the sample on said swab pad to the test reaction vessel of step (e)

(iii) to which vessel buffer solution and conjugate have already been added, by immersing the swab pad in the buffer solution in said test reaction vessel [contained therein], twirling the pad in said solution and leaving the pad immersed therein throughout the period of incubation set forth in step (e) (iii).

22 A method according to Claim 21 wherein the buffer solution is composed of aqueous 0.05 M tris HCl containing 2-5% of a detergent having a pH of about 7.0.

23 A method according to claim 20 wherein the concentration step comprises subjecting each individual water sample to high speed centrifugation followed by settling and removal by decantation or aspiration of supernatant water and it is followed by thoroughly stroking the residual solids with the swab pad of a swab comprising a handle and an affixed pad of fibrous material or foamed open pore material and delivering the sample on said swab pad to the test reaction vessel of step (e) (iii) to which vessel buffer solution and conjugate have already been added, by immersing the swab pad in the buffer solution and leaving the pad immersed therein throughout the incubation period set forth in step (e) (iii).

24 A method according to claim 23 wherein the buffer solution is composed of aqueous 0.05 M tris HCl containing 2-5% of a detergent having a pH of about 7.0.

25 A method for determining the concentration of at least one species, or serogroup of a species, of *Legionella* bacteria in environmental water suspected of being infected therewith, which method comprises the following steps:

(a) culturing an identified *Legionella* bacteria species, or serogroup of a species, to a desired size and harvesting therefrom cells of that species, or serogroup of a species, as a wet cell pellet;

(b) obtaining from the wet cell pellet an essentially protein-free embodiment of the characteristic O-carbohydrate antigen of that species, or serogroup of a species, of *Legionella* bacteria by a series of steps which comprises

(i) suspending the wet cell pellet in an alkaline solution and mixing to form a mixture;

(ii) adjusting the pH of the mixture to an acid pH with a strong acid;

(iii) separating the mixture from step (ii) into two layers, an upper layer and a lower layer;

(iv) removing the upper layer and adjusting its pH to approximate neutrality;

(v) adding to the approximately neutral pH upper layer from step (iv) a broad spectrum protease enzyme and digesting to destroy residual proteins;

(vi) adjusting the pH of the digested product from step (v) to an alkaline pH with a dilute aqueous alkaline solution of a weak base and;

(vii) separating out the essentially protein free O-carbohydrate antigen embodiment;

(c) coupling to a chromatographic affinity column through a spacer molecule the essentially protein-free O-carbohydrate antigen embodiment obtained in step (b);

(d) passing polyclonal antibodies to the *same Legionella* species, or serogroup of a species, as that cultured in step (a) over the chromatographic affinity column of step (c) to produce purified antigen-specific antibodies;

(e) preconcentrating an environmental water sample suspected of containing *Legionella* bacteria of the same species or serogroup of a species, as that cultured in step (a) by (1) adding thereto and mixing therewith an aqueous medium containing finely divided magnetizable particles which have and mixing therewith been precoated with purified antibodies from step (d) hereof, which antibodies tend to draw to themselves characteristic O-carbohydrate antigens of the same *Legionella* bacteria species or serogroup of a species as that cultured in step (a) from the bacteria in the sample and to react therewith to form conjugates, (2) subjecting the mixture of sample and magnetizable particles to the action of a local magnetic field, whereby they are caused to form a coherent mass, (3) decanting or aspirating off the water from the coherent mass and (4) subjecting the mass, in a known manner, to demagnetization and then to elution of the antigen-antibody conjugates from the particles; and

(f) performing an enzyme immunoassay upon the resulting eluate according to the following steps:

(i) coating a solid substrate with antigen-specific antibodies from step (d) hereof in an amount sufficient to provide a coating containing at least 0.05 μg per test of said antibodies and allowing the coated substrate to dry;

(ii) preparing a conjugate of an enzyme with the antibody-antigen conjugates in the eluate from step (e);

(iii) bringing enzyme-antibody-antigen conjugate containing from 0.2 to 2.0 μg per test of enzyme- antibody content into contact with a buffer solution and the coated solid substrate of step (i) in a test reaction vessel and incubating for a period of at least twenty minutes; and

(iv) decanting liquid from said vessel, washing, adding a colorimetric, chemiluminescent or bioluminescent material thereto, allowing color, chemiluminescence or bioluminescence to develop for up to about 5 minutes, measuring its intensity in any known manner and determining therefrom the concentration of the suspected *Legionella* species, or serogroup of a species, in the original water sample according to predetermined intensity/concentration standards which first correlate the measured intensity to the amount of O-carbohydrate antigen characteristic of at least said suspected species, or serogroup of a species, of *Legionella* bacteria present in the sample and then correlate that amount in turn to the concentration of the suspected *Legionella* bacteria species, or serogroup of a species, in CFU/ml, present in the water being tested.

26 The method of claim 25 in which the *Legionella* bacteria species, or serogroup of a species, in step (a) is a serogroup of *Legionella pneumophila* bacteria and the polyclonal antibodies of step (d) are polyclonal antibodies to bacteria of the same serogroup of *Legionella pneumophila.*

27 The method of claim 26 in which the bacteria of a serogroup of *Legionella pneumophila* are bacteria from serogroup 1.

30 A method according to claim 25 wherein the enzyme immunoassay is run as a sandwich assay.

31 A method according to claim 25 wherein the enzyme immunoassay is run as a competitive assay.

32 A method according to claim 25 wherein the coated solid substrate in step (f) (i) is the inner wall of a test tube, which test tube also serves as the test reaction vessel in steps (f) (iii) and f (iv).

33 A method according to claim 25 wherein the coated solid substrate in [of] step (f) (i) is selected from among coated solid inserts and coated beads.

34 A method according to claim 25 wherein the enzyme of the conjugate prepared in step (f) (ii) is horseradish peroxidase and a colorimetric agent, tetramethylbenzidine, is added in step (f) (iv).

35 A method according to claim 25 in which the buffer solution of step f (iii) is aqueous 0.05 M tris HCl containing 2-5% of a detergent having a pH of about 7.0.

APPENDIX B

DISCLOSURE OF APPLICATION SERIAL NO. 09/139,720
(Incorporated By Reference)

ICT Immunoassay for Legionella Pneumophila Serogroup 1 Antigen Employing Affinity Purified Antibodies Thereto

This invention relates to essentially protein-free carbohydrate and polysaccharide antigens separated from bacteria of the genus Legionella, and especially from serogroups and/or strains of Legionella pneumophila, including the O-polysaccharide antigen of L. pneumophila serogroup 1, and to the use of these antigens in the affinity purification of polyvalent antibodies to corresponding Legionella organisms. More particularly, the invention encompasses coupling the carbohydrate or polysaccharide antigen separated from a Legionella bacterium to an activated chromatographic column and using that column for affinity purification of the polyclonal antibodies to the same species, or the same serogroup of a species, of Legionella. The invention further encompasses the use of the affinity-purified polyvalent antibodies produced in immunochemical assays for the detection of Legionella-caused diseases such as Legionnaires disease and Pontiac fever in human patients and for the detection of environmental sources of Legionella infectious agents.

BACKGROUND

Legionnaires' disease, a pneumonia-like human infection caused by gram-negative bacteria of the genus Legionella, is virtually impossible to differentiate on a reliable clinical basis (involving assessment of patient symptoms without laboratory tests) from pneumonia and other similar lung infections. Because the disease may produce lung abscesses, infections in other bodily organs or bacteremia, and its mortality rate is significantly increased by delay in commencing appropriate therapy, there is a need for rapid and reliable diagnostic tests which has to date been only partially met. Stout, J.E. and Yu, V.L., "Legionellosis", 337, N. Eng.

J. Med. 682-687 (1997). Efforts to develop such tests have been hampered by the fact that there are a number of Legionella species, at least some of which are known to have a number of distinct serogroups.

Legionnaires' disease was first recognized in the summer of 1976 and a number of techniques for detecting Legionella ("L.") pneumophila, which is now known to account for some 90% of cases (Stout and Yu, supra), have been developed in the interim. In general, these tests are time-consuming and incapable of identifying more than one serogroup of the 15 serogroups known so far to fall within the L. pneumophila species. It should be noted, however, that, as reported by Stout and Yu, supra, more than 80% of reported cases of Legionnaires' disease are attributable to L. pneumophila serogroup 1 -- a fact which makes the development of a rapid, reliable immunoassay for that entity of particular importance and has led researchers to focus on this as a priority.

The early efforts to establish the identity of the causative agent of Legionnaires' disease depended largely upon culturing the bacteria for 5 to 6 days and examining the culture microscopically. Efforts to speed up the identification process led to numerous immunochemical tests of varying sensitivity and specificity including, inter alia, the presently commercially available EQUATE™ radioimmunoassay ("RIA"), and the Binax enzyme immunoassay ("EIA"), both of which are sold in kit form by applicants' assignee, Binax, Inc. As indicated by Hackman, B.A. et al. in "Comparison of Binax Legionella Urinary Antigen EIA Kit with Binax RIA Urinary Antigen Kit for Detection of Legionella pneumophila Serogroup I Antigen", 34 J. Clin. Microbiol. 1579-1580 (1996), both of these assays were found to be specific for L. pneumophila Serogroup I antigen. The reported sensitivity of the EIA was 77%; that of

the RIA was higher. The article indicates that each can be performed within "less than 3 h[ours] from beginning to end"; in Binax's own tests each requires at least 2 1/2 hours to perform. More information about this EIA assay appears in Kazandjian, D. et al., "Rapid Diagnosis of Legionella pneumophila Serogroup I Infection with Binax Enzyme Immunoassay Urinary Antigen Test", 35 J. Clin. Immunobiol. 954-956 (1997).

BRIEF DESCRIPTION OF THE INVENTION

Perhaps the most significant advantage of the immunochromatographic test ("ICT") described herein is its ability to give a test result within a 15-minute time span for the presence or absence of L. pneumophila Serogroup I (or its antigen), which result is of high specificity and sensitivity. The speed with which this test can be reliably conducted to yield a result of high specificity and sensitivity is believed to be due to the strongly reactive nature of the affinity purified antibodies prepared in accordance with this invention. The superior reactive properties of these antibodies, in turn, is believed to be attributable to the use for affinity purification of the novel, essentially protein-free O-polysaccharide antigen of L. pneumophila serogroup 1 which is also a part of this invention.

Another ICT test has been made possible by the separation, according to this invention, of an antigen of L. pneumophila serogroup 5 which is of non-proteinaceous, carbohydrate nature and is common to multiple serogroups of L. pneumophila. The presence of such a common antigen has been suggested in the scientific literature; see e.g., Nolte, F.S. et al., "Electrophoretic and Serological Characterization of the Lipopolysaccharides of Legionella pneumophila", 52 Infection and Immunity 676-681 (1986); Otten, S. et al., "Serospecific

Antigens of Legionella pneumophila", 167 J. Bacteriol. 893-904 (1986); Barthe, C. et al., "Common Epitope on the Lipopolysaccharide of Legionella pneumophila Recognized by a Monoclonal Antibody", 26 J. Clin. Microbiol. 1016-1023 (1988); Knirel, Y.A. et al., "The Structure of the O-specific Chain of Legionella pneumophila Serogroup I", 227 Eur. J. Biochem. 239-245 (1994). Heretofore there has been no clear report of any separation of such an antigen for use in developing a useful broad spectrum immuno-chemical assay.

This invention comprises the extraction from any Legionella bacterium, and especially from a bacterium of any of the serogroups of L. pneumophila of an essentially protein-free polysaccharide or carbohydrate antigen, the preparation of a conjugate of this antigen with a spacer protein, the coupling of the conjugate to an affinity column, the use of that column for the purification of polyvalent antibodies to the corresponding Legionella bacterium, such as a bacterium of a serogroup of L. pneumophila and the use of the antibodies thus purified in an ICT immunoassay for detecting Legionella bacteria or their antigens, including antigens of L. pneumophila serogroups or their corresponding bacteria, as more specifically described hereinafter.

In particular, the invention includes the separation of an essentially protein-free O-polysaccharide antigen specific to L. pneumophila serogroup 1, its use in the affinity purification of the polyvalent antibody specific to the same microorganism and the use of that affinity purified antibody in an ICT immunoassay of high specificity and high sensitivity that is performable within 15 minutes.

In another embodiment, the invention includes the separation from L. pneumophila serogroup 5 of an essentially protein-free carbohydrate antigen, and its use in the affinity

purification of the polyclonal antibody specific to the L. pneumophila serogroup 5 antigen (i.e., an antibody that was obtained from a rabbit immunized with the said antigen); this antibody showed an ability to cross-react with antigens of the L. pneumophila serotypes 1, 2 and 4 in addition to the antigen of serotype 5.

DESCRIPTION OF THE DRAWINGS

Figure 1 and its related Figures 1A, 1B and 1C hereof show the structure of a typical ICT device which is suitably adapted to perform the L. pneumophila serogroup 1 specific assay, as described in Examples VII, VIII and IX hereof.

Figure 2 hereof shows the results of Western blot analyses of phosphate-buffered saline extracts of L. pneumophila serogroups 1, 2, 4 and 5 with L. pneumophila serogroup 5, essentially protein-free carbohydrate antigen-affinity-purified polyclonal antibody specific to serogroup 5 antigen, which analysis is described in Example X hereof.

DETAILED DESCRIPTION OF THE INVENTION

Previous experience in the art, including experience with the Binax RIA assay for L. pneumophila Serogroup 1 antigen sold under the trademark EQUATE and the Binax EIA assay for the same antigen, has shown that antigens of the Legionella species, including antigens of various L. pneumophila serogroups, are more conveniently detectable in specimens from the urine of patients infected with a Legionella microorganism than in specimens of blood, sputum or other fluids. This is in part because the Legionella antigens usually appear in urine within 1-3 days after infection of a human patient whereas their appearance at a detectable level in

blood may occur later, and also in part because patients infected with Legionnaires' disease often do not produce much sputum. The ICT test which forms a part of this invention can be configured to run on blood, sputum or some other fluid such as cerebrospinal fluid, or on aqueous samples of environmental origin. It is noted that urine is generally the preferred sample fluid for diagnosis of human patients because it can be obtained non-invasively and easily, even in the doctor's office, and it is not as readily contaminated with other micro-organisms, e.g., oral microflora present in sputum, which are innocuous but may affect the results obtained.

Broadly speaking, the ICT test for L. pneumophila serogroup 1 antigen that is specifically a part of the present invention may be designed to be run in any known disposable ICT device disclosed in the art. Preferably, the test is conducted using an ICT device of the type disclosed in copending U.S. Application Serial. No. 07/706,639 (now U.S. Patent 6,168,956) of Howard Chandler, or one of its continuation-in-part applications, all assigned to Smith-Kline Diagnostics, Inc. but exclusively licensed to Binax, Inc. in a wide area of applications that includes the present diagnostic field -- i.e., diseases of the respiratory system. The device is suitably impregnated with the affinity purified polyvalent antibodies herein disclosed which are specific to the antigen of L. pneumophila serogroup 1 antigen. Positive results of the assay are shown by the appearance of a color upon reaction of suitably labeled antibodies with the antigen. Suitable labels may be any of those known in the art to produce visible color when antibodies conjugated thereto react with antigen, including finely divided metallics and various other labeling materials. Colloidal gold is the preferred label.

The invention contemplates that essentially protein free carbohydrate or polysaccharide antigens may similarly be separated from each of the Legionella bacteria, including from bacteria of other Legionella species and from bacteria of other serogroups of L. pneumophila, that such antigens may similarly be utilized in the affinity purification of polyvalent antibodies to the corresponding Legionella species or serogroup bacteria and its antigen, and that these affinity-purified antibodies may be utilized in ICT and other immunoassays as specifically disclosed herein for affinity-purified antibodies to L. pneumophila serogroup 1.

Preliminary to the preparation of the device is obtaining the antibodies and effecting their affinity purification. The antibodies that may be used in this invention are conventional polyvalent (also called "polyclonal") antibodies obtained by the well known process of immunizing a rabbit, goat or other animal to a Legionella antigen, e.g., L. pneumophila antigen of known serogroup and bleeding the animal after the passage of an appropriate time period to obtain serum containing the desired antibodies. See, e.g., Cherry, U.B. and McKinney, R.M., pp. 91-104 in Jones, G.L. and Herbert, G.A. (Eds.), "Legionnaires'" the disease, the bacterium and the methodology, (Center for Disease Control, Atlanta, 1979). In this invention, relative to the ICT immunoassay for L. pneumophila serogroup 1 disclosed herein, the rabbit or other animal is immunized to L. pneumophila serogroup 1 antigen and the polyvalent antibodies recovered from its blood are antibodies to L. pneumophila serogroup 1.

The polyvalent antibodies to be used in this invention are further subjected to affinity purification on a specially prepared chromatographic column which employs the essentially protein-free carbohydrate or polysaccharide antigen of the same bacterium against which the antibodies are reactive as the purifying agent for them.

Preliminary to the affinity purification of the antibodies, it is therefore necessary according to this invention to prepare an essentially protein free purified polysaccharide or carbohydrate antigen from a culture of known Legionella bacteria of the desired species or serogroup of a species.

The following examples I and II explain how the purified protein free polysaccharide or carbohydrate antigen is obtained.

Example I

Bacteria of L. pneumophila Serogroup 1 (strain Philadelphia-1) were obtained from Centers for Disease Control and Prevention (Atlanta, GA) and cultured on charcoal-yeast extract agar plates obtained from Northeast Laboratory (Waterville, ME) for a period of 72 hours at 37°C. Cells were harvested with phosphate-buffered saline ("PBS") at pH 7.2 containing 0.2% of NaN_3 and collected by centrifugation at 8000 rpm for 25 minutes. The resulting cell pellet was stored at -20°C. until used.

Wet cells from this pellet were suspended in 20 ml. 0.1 M NaOH per gram of wet cells and stirred at room temperature for 45 minutes. The pH of the solution was then adjusted with concentrated acetic acid to 3.0 and the solution was subjected to centrifugation at 8000 rpm for 20 minutes. The supernatant from this step was then neutralized with aqueous NaOH and dialyzed against distilled water.

The resulting dialyzate was concentrated 10 times on a rotary vacuum evaporator and then sonicated for 5 minutes in an ultrasonic bath.

Proteinase K, in a concentration of 0.2 mg. per ml. of the concentrated product, was added to digest the remaining proteins and the mixture was incubated at 40°C. overnight. The

next step was the addition of further Proteinase K, in a concentration of 0.1 mg. per ml., to the mixture, followed by further overnight incubation at 40°C. This second incubation was followed by concentration of the product on a rotary evaporator to a small volume, adjustment of its pH to 10-11 with 0.2% triethylamine and application of the thus-treated mixture to a column of Sephacryl S-200 from Pharmacia, equilibrated with 0.02% triethylamine. Material eluted in the first peak was pooled, adjusted with 0.1 N HCl to approximately neutral pH and dialyzed against distilled water for 18 hours, followed by lyophilization.

The yield of O-polysaccharide antigen from 16.5 grams of wet cells of L. pneumophila serogroup 1 strain Philadelphia-1 was 62 mg.

It should be noted that in place of Proteinase K, any broad spectrum protease enzyme preparation may be used. What is important in this procedure is the elimination of protein from the antigen to the maximum possible extent within reasonable limits of time feasibility.

Example II

Example I was repeated using L. pneumophila serogroup 5 strain Dallas IE as the bacteria in the culture step. 11.5 wet grams yielded 21 mg. of a carbohydrate antigen. At a later time, the procedure was again repeated, this time using L. pneumophila serogroup 5 (strain U8W) as the bacteria in the culture step.

It is within the scope of this invention to repeat the culturing and extraction steps as herein described on any other Legionella bacterium and especially on bacteria of any other of the serogroups of L. pneumophila, i.e., on any of serogroups 2, 3, 4 and 6-15 inclusive, to obtain a carbohydrate antigen essentially free of proteinaceous material.

In order to couple the essentially protein free O-polysaccharide product of Example I or the carbohydrate of Example II to a chromatographic column for use in affinity purification of polyvalent antibodies to the corresponding bacterium from which the polysac-chloride or carbohydrate antigen was extracted, it is necessary to complex it with a protein spacer molecule suitably prepared to ensure that it will stably bond to both the polysaccharide or carbohydrate antigen and the column and will itself remain inert during the affinity purification step. A modified bovine serum albumin ("BSA") was chosen as the preferred spacer molecule and was prepared as in Example III:

Example III -- Preparation of Protein Spacer Molecule

An 0.5M aqueous solution of hydrazine dihydrochloride from Aldrich Chemical Co., Inc. (Milwaukee, WI) was prepared and its pH was adjusted to 5.2 with dry NaOH. The solution was then mixed with dry BSA from Sigma Chemical Co. to a final concentration of 25 mg. per ml. of BSA. After the BSA was completely dissolved, N-(dimethylaminopropyl)-N^1-ethylcarbodiimide hydrochloride obtained from Fluka Chemical Co. (St. Louis, MO) was added to a final concentration of 2.5 mg. per ml. The mixture was stirred overnight at ambient temperature and then dialyzed for a period of about five days against distilled water at 4°C, with daily changes of the water.

In lieu of this modified BSA, it is contemplated that other appropriate spacer molecules may be used.

The conjugation of the O-polysaccharide antigen from serogroup 1 of L. pneumophila to the spacer molecule was conducted as follows:

Example IV -- Conjugation of O-polysaccharide Antigen to Spacer Molecules

L. pneumophila serogroup 1 essentially protein-free O-polysaccharide antigen was dissolved in distilled water in a concentration of 4-5 mg/ml and the pH was adjusted to 5.0 with 1 M HCl. The modified BSA solution prepared as in Example III was adjusted to pH 5.0 with 1 M HCl and slowly added, in a weight ratio of 4-to-1 of the O-polysaccharide antigen solution, to the latter. After 5 minutes of stirring, about 100-200 mcl of distilled water containing the N-(dimethylamino-propyl-N^1-ethylcarbodiimide hydrochloride referred to in Example III was added in a weight ratio of 1:2 to the O-polysaccharide antigen/BSA solution. The resulting mixture was stirred at ambient temperature overnight.

To separate the conjugate of the O-polysaccharide antigen with modified BSA from any unreacted materials present, the reaction mixture was chromatographed on a Sepharose CL-4B column equilibrated with a buffer of 0.1 M NaH_2PO_4:0.5 M NaCl. All of the chromatography fractions were subjected to testing for antigen activity using the commercial Binax EIA test referred to above. All fractions that showed antigen activity in the tested were pooled in and used for affinity column preparation as shown in Example V.

Example V -- Coupling of Conjugate to Activated Chromatographic Column

An immunoadsorbent gel was prepared by conventionally activating Sepharose CL-4B with cyanogen bromide. The ligand of O-polysaccharide antigen with modified BSA, prepared as in Example IV, was coupled to it using procedures known in the art, e.g., as described in Hermanson, G.T., et al. in Immobilized Affinity Ligand Techniques, 53-56 (Academic Press, Inc. 1992). The gel was then packed in a column and washed successively with 5-10 volumes per volume of gel with PBS of pH 7.2, triple strength PBS of pH 7.2 and 0.2 M glycine-HCl of pH 2.5. The resulting activated column was used, as described below, for affinity purifi-

cation of polyvalent antibodies to L. pneumophila serogroup 1 antigen. The column after elution should be stored in PBS or anther neutral buffer until used.

Instead of cyanogen-bromide activated Sepharose, spherelose and various commercially available activated columns could be used in this step.

Example VI -- Affinity Purification of Antibodies

The activated column described in Example V was used for the affinity chromatography of rabbit-anti-L. pneumophila serogroup 1 polyvalent antibodies to L. pneumophila serotype 1 antigen according to the method described by Harlow E. and Lane, D. in Antibodies: A Laboratory Manual at 313-315 (Cold Spring Harbor Laboratory, 1988).

Elution of the antibodies from the column was effected with 0.2M glycine-HCL buffer of pH 2.5. Alternative eluants such as 3M NaSCN or 0.1M Et_3N could be substituted.

These affinity-purified antibodies were utilized in an ICT test specific to L. pneumophila serogroup 1 as described in the following example.

Example VII -- ICT Device and Its Preparation

A. Preparation of Test Device:

A test device comprising a hinged cardboard housing equipped with a window to allow the viewing of both the test results and control results was prepared as shown in Figure 1. The device has a recess into which is placed a preformed plastic swab well for receiving the sample-wetted swab on the right-hand (labeled 1 in the drawing). An overlabel shown in Figure 1A is then placed over the entire right-hand side of the device. The overlabel has been equipped with two holes -- a lower one (marked B on Figure 1A) into which the saturated swab is to be inserted and an upper one (marked A on Figure 1A) toward which the swab will

be pushed after insertion thereof into the hole B. The position of the overlabel with its holes A and B, and the swab well cooperate to hold the swab in a proper position during the assay and to promote the expulsion of sorbed liquid from the swab.

A preassembled test strip (marked C on Figure 1) described below, is inserted into the recess (labeled 2 on Figure 1) and held in place by an adhesive applied to the bottom thereof. An overlabel shown in Figure 1B is placed atop the left-hand side. It has been equipped with a single hole (marked D in Figure 1B) which mates to the right-hand side hole A when the device is closed for performance of the assay.

The assembled device is stored in a sealed pouch with desiccant until it is used. Prior to sealing the pouch and storing, a lightly adhesive tape is placed on the outer edge of the right-hand half of the device.

B. Construction and Preparation of the Preassembled Test Strip

Figure 1C shows the construction of the preassembled strip. It is comprised of a conjugate pad of sorbent material in which a conjugate of gold particles and the affinity-purified rabbit anti-Legionella pneumophila serogroup 1 antibodies described above have been impregnated. In contact with this pad is a nitrocellulose pad onto which a capture line for the sample which reacts with the conjugate has been established by embedding a stripe of affinity-purified rabbit anti-L. pneumophila serogroup 1 antibodies, prepared as described above. The nitrocellulose pad also has a downstream control line established by striping the pad with goat anti-rabbit immunoglobulin (IgG). Following the nitrocellulose pad, the strip is ended by an absorbent pad which serves as a reservoir for liquid. All of these pads are backed by an adhesive strip when the device is ready to ship.

The conjugate pad is normally made from non-woven polyester or extruded cellulose acetate. To prepare this pad for use in the assay, gold particles of 50 nm. diameter are conjugated to affinity-purified rabbit anti-Legionella pneumophila serotype 1 antibodies prepared as described above. The conjugation is effected using a known method such as that described by DeMay in Polak, J.M. and Van Norden, S. (Eds.), Immunochemistry: Modern Methods and Application, (Wright, Bristol, England, 1986). The gold conjugate particles are mixed with a drying agent consisting of aqueous 5mM sodium tetraborate of pH 8.0 containing 1.0% BSA, 0.1% Triton X-100, 2.0% Tween 20, 6.0% sucrose and 0.02% sodium azide. The pad is heated sufficiently to remove all of the liquid present and stored in a low-humidity environment pending assembly of the test strip. These pads and their treatment are especially chosen so that the pads will hold the dry conjugate and will release it only when later wetted by sample.

The nitrocellulose pad is first treated by embedding a stripe of affinity purified rabbit anti-L. pneumophila serotype 1 antibodies in a first portion thereof, using a carrier solution of phosphate buffered saline. These antibodies act as the capture line. In a second portion of the pad downstream of the first one in the assembled test device, the control line is established by striping goat anti-rabbit IgG in the same carrier solution on the surface of the pad. The nitrocellulose pad is then subjected to desiccation at 18-25° C to promote permanent absorption of the protein stripes thereto.

The absorbent pad used is of a commercially available cellulosic material sold under the name Ahlstrom 939. This pad requires no special treatment.

C. Kit Preparation

As sold in commerce, the test device containing the finished test strip is assembled. In practice, a number of devices are packaged with a commensurate number of swabs fashioned from fibrous Dacron and a bottle of "Reagent A" equipped with a top adapted to deliver Reagent A dropwise. "Reagent A" is a solution of 2.0% Tween 20, 0.05% sodium azide and 0.5% sodium dodecyl sulfate in a 0.05 M sodium citrate-sodium phosphate buffer of pH 6.5. Positive and negative controls are also included in each kit.

The use of the finished test devices to identify L. pneumophila serogroup 1 antigen is illustrated in the following example VIII:

Example VIII -- Conducting the ICT Test for L. pneumophila Serogroup 1 Antigen

In practice, the swab furnished with each device is dipped into the liquid sample, completely immersing the swab head. The use of the swab to act as a filter for undissolved solids, semisolids and colloids present in liquid biological samples such as urine, blood, lymph, etc. and also in liquid environmental samples is the subject of copending Application Serial No. 09/044,677 of Norman Moore and Vincent Sy filed March 19, 1998, which ~~will shortly be~~ is assigned to Binax, Inc. The swab is inserted into the hole at the bottom of the device (hole B of Figure 1A) and gently pushed upward so that the swab tip is visible in the top hole (hole A of Figure 1A). The Reagent A vial is held vertically above hole B and two drops of Reagent A are slowly added. The adhesive liner is then immediately peeled from the right edge of the device and the device is closed and securely sealed, thus pressing the swab in the swab well against the gold conjugate pad. After 15 minutes, the result can be read in the window of the device. A negative sample -- i.e., one containing no L. pneumophila serogroup 1 antigen -- will exhibit only the control line in the top half of the window. A positive sample containing

the target antigen will show two lines, the lower one of which is the patient (or sample) line; even a faint sample line indicates the presence of the target antigen in the sample. If no line appears in the window after 15 minutes, or only a sample line appears in the lower part of the window, the test is invalid and must be repeated.

Using the procedure described above, the devices prepared as described in Example VII were tested in the ICT procedure just described against 300 patient urine samples, 100 of which had been previously diagnosed as having L. pneumophila serogroup 1 infection.

These ICT tests according to this invention were conducted under circumstances such that the previous diagnoses were unknown to personnel performing the ICT tests. Overall, 98% of the ICT tests agreed with the previous positive diagnoses. Also overall, 98% of the urine samples previously diagnosed as negative for L. pneumophila serogroup 1 antigen gave results in agreement therewith when tested by the ICT procedure described herein, using the ICT device described in Example VII.

Example IX -- Use of the ICT to Test Environmental Samples

Applicability of this same test to environmental samples suspected of containing L. pneumophila serogroup 1 was also investigated as follows:

Water was seeded with L. pneumophila serogroup 1 bacteria obtained from a commercial source. The mixture was concentrated by filtering through a 0.22 μm filter. A swab dipped in the sample was applied to the device, the device was closed and the assay was allowed to proceed. A positive result was observed within less than 15 minutes.

Example X - Western Blot Immunoassay For Detection of Cross-Reactive Carbohydrate Antigens of L. Pneumophila Serogroups 1, 2, 4 and 5

In order to perform the Western Blot immunoassay using a kit purchased from Bio-Rad Laboratories, L. pneumophila serogroup 5 cells were cultured as in Example II. A suspension of these cells was solubilized with 1% sodium dodecylsulfate in the presence of 10 mM mercaptoethanol at 100° C. for 5 minutes. The solubilized cells were treated with protease K and then subjected to electrophoretic separation of protein according to standard procedures provided by Bio-Rad.

The carbohydrate antigen from L. pneumophila serogroup 5 was conjugated to the spacer molecule described in Example III hereof in the manner described in Example IV and applied to an activated Sepharose column as described in Example V. This column was then used for the affinity purification of polyvalent rabbit antibodies specific to the carbohydrate antigen of L. pneumophila serogroup 5 (which were conventionally obtained from serum of a rabbit previously injected with the protein-containing of L. pneumophila serogroup 5) using the procedure of Example VI.

The Western immunoblot analysis was performed using a reagent kit from Bio-Rad and according to directions from this manufacturer. Briefly, the PBS extract of cells of L. pneumophila antigens 1, 2, 4 and 5 was subjected to the SDS-PAGE in 12% polyacrylamide gel blocked with 1% BSA with PBS transferred onto a nitrocellulose membrane. After this step, the membrane was incubated with affinity purified antibodies specific to carbohydrate of L. pneumophila serogroup 5. The membrane, washed as recommended by the manufacturer, and incubated with horseradish peroxidase conjugated to goat-anti-rabbit antibodies provided

by Bio-Rad. After washing, the membrane was developed with a substrate system of 0.02. 4-chloro-1 naphthol and 0.0012 M N, N-dimethyl-p-phenylene-diamine monohydrochloride in 0.1 M sodium citrate buffer of pH 6.9 containing 2.9 mM of hydrogen peroxide. Figure 2 hereof shows the Western blot assay results compared with that of the prestained SDS-PAGE standard (in Lane 5) for the affinity purified antibodies of serogroup 5 of L. pneumophila against PBS extracts containing antigens of L. pneumophila as follows:

Lanes 1 and 7--L. pneumophila serogroup 2 (strain Togus-1)
Lanes 2 and 8--L. pneumophila serogroup 4 (strain Los Angeles-1)
Lanes 3 and 6--L. pneumophila serogroup 1 (strain Philadelphia-1)
Lanes 4 and 9--L. pneumophila serogroup 5 (strain U8W).

It is pointed out that the affinity purified antibodies for Lanes 1-4 were affinity purified on a column to which carbohydrate antigen from L. pneumophila serogroup 5 (strain U8W) was attached while those for Lanes 6-9 were affinity purified in the same manner on a column having attached carbohydrate antigen of L. pneumophila serogroup 5 (strain Dallas IE).

Figure 2 clearly demonstrates that affinity purified antibodies as herein disclosed of L. pneumophila serogroup 5 react with antigens of L. pneumophila serogroups 1, 2, and 4 in addition to those of serogroup 5.

An ICT assay as described above in which affinity purified antibodies from L. pneumophila serogroup 5 are substituted for affinity purified antibodies from L. pneumophila serogroup 1 is contemplated.

Those skilled in the art of immunochemistry generally, and especially those skilled in immunoassays, will recognize that other materials and ingredients and at times, other procedural steps, can readily be substituted for those specifically recommended herein. A vast

array of literature, both patent and non-patent, discusses the design and use of reliable, one-time-use, disposable immunoassay test devices that could be substituted for the preferred ICT device described and recommended herein. It is not intended that the present invention should be limited with respect to substitutable assay devices, materials, ingredients or process steps except insofar as the following claims may so limit it.